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Endogenous Retinoids in Rat Epididymal Tissue and Rat and Human Spermatozoa[1]

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ABSTRACT

Recent work has demonstrated high levels of retinoid binding proteins in rat epididymis, and a lumenal retinoic acid binding protein has been purified. These findings suggested that vitamin A may be involved in spermatozoal maturation in the epididymis. We further addressed this question by quantifying retinol, retinyl esters, and retinoic acid isomers from perfused epididymal tissue, from rat testicular and epididymal spermatozoa, and from human ejaculate sperm.

HPLC showed vitamin A levels to be higher in caput than in corpus or cauda tissue. Retinoic acid and 9-*cis*-retinoic acid were found to be graded from lowest levels in caput to highest in cauda.

Spermatozoa from caput epididymidis and enriched testicular spermatozoa were found to have higher levels of vitamin A than did spermatozoa from corpus or cauda epididymidis. Spermatozoal retinyl esters had acyl substituents similar to those seen in whole epididymis, and diminished in quantity in sperm from distal segments.

Human ejaculate sperm were found to retain high levels of retinyl palmitate and stearate. Retinol and retinoic acid were only marginally detectable in human sperm.

Retention of retinoids in mature spermatozoa suggests roles for vitamin A in spermatozoal reproductive physiology beyond the epididymal stage.

INTRODUCTION

It has long been established that vitamin A plays a role in epididymal physiology, as vitamin A deprivation in rats has shown to result in replacement of the epididymal epithelium with a stratified squamous keratinizing epithelium [1]. The fact that the epididymis contains retinoid binding proteins is consistent with a role for vitamin A in the epididymis. RIA for cellular retinol-binding protein (CRBP, one of the intracellular vitamin A binding proteins) has revealed that CRBP levels are higher in epididymis than in over 30 other tissues examined in rats, including testis [2, 3]. The initial segment and proximal caput contains 90% of epididymal CRBP, making the proximal epididymal CRBP level higher than even that of liver tissue, where vitamin A is stored [4, 5]. Immunolocalization studies have revealed that CRBP is found predominantly in the cytoplasm of principal cells, with little or no immunostaining in spermatozoa, peritubular cells, or other epithelial cells [6, 7]. RIA for cellular retinoic acid-binding protein (CRABP, one of the intracellular proteins that binds all-*trans*-retinoic acid, an active metabolite of retinol) has revealed the presence of high levels of this retinoid binding protein, with almost 90% of the CRABP detected in the distal epididymis [4]. Immunolocalization results have shown that CRABP is present in testis germ cells, the cytoplasm of principal cells in caput epididymidis, stereocilia in principal cells in the cauda, and in spermatozoa throughout the epididymis [6].

Possible evidence for retinoid trafficking between tissue and spermatozoa in the rat has arisen through discovery that a major androgen-dependent protein that is secreted into the epididymal lumen in the initial segment and proximal caput epididymidis [8, 9] is able to bind retinoic acid in vitro [10, 11]. The two forms of this protein, called B and C [9], which differ only by three additional amino acids at the N-terminus of the latter, have sequence homology with retinol binding protein and other members of the α-$_2\mu$-globulin superfamily [12, 13].

On the basis of the high levels of retinoid binding proteins, it has been suggested that vitamin A and its metabolites may have roles in sperm maturation, possibly through the involvement of vitamin A in regulation of epididymal maturational protein synthesis and secretion [5]. It has further been suggested that the epididymis may play a more direct role in vitamin A-related spermatozoal differentiation by means of the transport of retinoids between the epididymal epithelium and spermatozoa [10, 11, 13].

In the present study, endogenous levels of vitamin A and some of its metabolites have been directly determined in rat epididymal tissue and spermatozoa in order to aid in elucidating the roles of vitamin A in epididymal physiology. A preliminary examination of retinol esterification activity in epididymal spermatozoa has been accomplished. Finally, endogenous vitamin A in human sperm from ejaculates has been examined to assess the applicability of our findings to human physiology.

MATERIALS AND METHODS

Chemicals, Solvents, and Enzymes

Neutral alumina and HPLC-grade dimethylformamide were obtained from Aldrich Chemical Co. (Milwaukee, WI). All

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other solvents were B & J brand, of HPLC grade or better (Baxter, Stone Mountain, GA). Clinical-grade dextran, DNase I from bovine pancreas, cholesterol esterase from bovine pancreas, BSA, and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Collection of Testis, Epididymal, and Vas Deferens Tissue

Sprague-Dawley rats (275–350 g, Harlan Co., Indianapolis, IN), were killed by decapitation. Testes (1.7–2.0 g, perfused), epididymides, and vasa deferentia were removed, perfused with ice-cold PBS, pH 7.4, and trimmed free of fat in ice-cold PBS.

Preparation of Rat Epididymal Sperm

Sperm were obtained from epididymides minced in PBS as previously described [14]. After filtration through eight layers of gauze, epididymal sperm were purified by a dextran wash [15] modified by the use of a step gradient of 10% dextran (30 ml) layered above 15% dextran in PBS (7.5 ml, 4°C). Sperm were applied to the gradient in 9 ml PBS and centrifuged as described by Tulsiani et al. [15] at 4°C. Purified sperm were diluted to 50 ml with ice-cold PBS and centrifuged (800 × *g*, 15 min, 4°C). Supernatants were discarded.

Distal cauda spermatozoa were obtained by retrograde flush via the vas deferens, then purified by dextran step gradient.

Enriched Spermatid and Testis Sperm Preparation

Perfused testes were minced in PBS after removal of capsules, shaken for 20 min, and centrifuged (50 × *g*, twice for 2 min each). Supernatants were pooled and filtered through Spectramesh (Spectrum Medical, Los Angeles, CA) 114-μm polypropylene filters. DNase I was added (100 μg/ml). The suspension was incubated 15 min at 21°C, loaded into a Beckman (Palo Alto, CA) model J-21B elutriator with a JE-6 elutriator rotor, and filtrate-fractionated as described [16, 17]. Testis spermatozoa collected in the first 200-ml elutriator effluent were collected on a Spectramesh 20-μm nylon filter and washed with PBS. Spermatozoa were backflushed from the filters with PBS and centrifuged (800 × *g*, 15 min).

An enriched preparation of round spermatids from testes of two rats was prepared as previously described [18].

Human Sperm Preparation

Human sperm were obtained from ejaculates from three individuals following procedures approved by the Vanderbilt Committee for the Protection of Human Subjects. Samples were allowed to liquify. Any remaining pieces of coagulum were removed before dilution with PBS and separation of sperm from seminal plasma by the modified dextran wash procedure.

Photomicrography of Sperm Preparations

Photomicrography was performed at 200× or 400× magnification, with an Olympus model BHS light microscope with Olympus (Overland Park, KS) model PM-10ADS photomicrographic system, using Kodak Tri-X pan 400 35-mm film (Eastman Kodak, Rochester, NY).

Homogenization, Extraction, and Fractionation of Retinoids

Extraction of retinoids was accomplished by combining and modifying methods previously described [19, 20] and was performed under nitrogen and dim yellow light. Minced tissue or sperm in 6–8 ml of ethanol containing 0.01% di-*t*-butyl-hydroxytoluene (BHT) were flushed with a stream of nitrogen and homogenized with a Polytron PT-3000 (Brinkman Instruments, Westbury, NY) at 29 000 rpm for 60 sec. One volume of 4.25 M NaCl with 0.025 M NaOH was added before extraction (two times) with one volume of hexane. Hexane phases were pooled and evaporated, and residues were dissolved in 0.5 ml hexane for fractionation by alumina chromatography [21, 22] on 10% water-deactivated alumina columns (0.2 g). Samples were loaded, washed with hexane (5 ml), and eluted with 2% ether/98% hexane (ester fraction) or 50% ether/50% hexane (retinol fraction). Fractions were evaporated and dissolved in dimethylformamide (ester fraction) or 92:8 hexane:dioxane (retinol fraction) for analysis.

The remaining aqueous phases were acidified and extracted as described [19]. After evaporation of the pooled hexane phases, the residues (retinoic acid fractions) were dissolved in 92:8:0.1 hexane:dioxane:acetic acid for analysis.

Retinoid Standards for HPLC

Retinoic acid, 13-*cis*-retinoic acid, 9-*cis*-retinal, retinol, 13-*cis*-retinol, and retinyl acetate standards were obtained from Sigma. Dehydroretinol and dehydroretinal were generous gifts from Hoffman-LaRoche (Nutley, NJ). Retinyl palmitate was obtained from Fluka Chemical Corp. (Ronkonkoma, NY). Other necessary retinyl ester standards were synthesized [20] and kindly supplied by Dr. T. Quick or Mr. K. Rigtrup of Vanderbilt University, or synthesized from retinol and the respective acyl chloride or anhydride (Nu Chek Prep, Elysian, MN) in triethylamine and purified by alumina chromatography [21, 22].

A mixture of all-*trans*-retinyl acetate and 13-*cis*-retinyl acetate was prepared by a 5-min exposure of the all-*trans*-standard to room light. Dehydroretinoic acid and 9-*cis*-retinoic acid standards were prepared from dehydroretinal and 9-*cis*-retinal by oxidative esterification [23]. Resulting methyl esters were retained as standards or hydrolyzed to the free acid standards at 21°C, with 0.3 M LiOH in 85:15 ethanol:water. After extraction of unsaponified material, the solution was acidified. The acids were extracted into petroleum ether.

Methyl esters of all-*trans*- and 13-*cis*-retinoic acid were prepared by reaction of the acids with diazomethane [24].

Endogenous Retinoid Analysis by HPLC

HPLC equipment included an ISCO (Lincoln, NE) model 2350 pump and model 2360 gradient programmer, and a V^4 absorbance detector with a Hewlett-Packard 3390A (Avondale, PA) or Spectra-Physics SP4270 (San Jose, CA) integrator. Retinol and retinoic acid were analyzed on a Whatman Partisil 5, 4.6 mm × 25 cm, 5-μm particle silica column (Hillsboro, OR) with a Supelco LC-Si 2-cm guard column (Supelco, Inc., Bellefonte, PA). The mobile phase was 92:8:0.1 hexane:dioxane:acetic acid (flow rate of 2 ml/min unless stated otherwise). Retinol was detected at 325 nm or 350 nm. Retinoic acid isomers were detected at 352 nm. Retinyl esters were detected at 325 nm after elution in 100% dimethylformamide (1 ml/min) from a Supelco LC-18 4.6 mm × 15 cm, 3-μm particle column, with Supelco LC-18 2-cm guard column.

Controls for Extraction Efficiency

Recovery of endogenous retinyl esters was estimated by recovery of retinyl heptadecanoate, added to triplicate sperm and tissue samples before homogenization.

Retinol and retinoic acid extraction efficiencies were determined by recovery of [15-^{3}H]retinol [25] or [11,12-^{3}H]retinoic acid (NEN, Wilmington, DE) added to sperm or tissue preparations. Recovery was assayed in evaporated extracts by liquid scintillation counting (counting efficiency, 29%).

Retinol Oxidation Control

Retinol (8100 picomoles, an amount greater than that observed in any tissue extract) was added to duplicate tubes, each containing 6 ml ethanol with 0.01% BHT. The solution was homogenized, extracted, and analyzed for retinoic acid by HPLC.

Retinoic Acid Isomerization Control

All-*trans*-retinoic acid (80 picomoles, more than that obtained from any extract) was added to a tube containing 8 ml ethanol with 0.01% BHT. The solution was homogenized, extracted, and analyzed for retinoic acid isomers by HPLC.

Derivatization of Endogenous Retinoids

Material from a cauda epididymal tissue extract that eluted at retention times of all-*trans*-, 13-*cis*-, and 9-*cis*-retinoic acid, were collected as they exited the HPLC. Fractions were evaporated, then reacted with 200 μl of 0.5 M diazomethane in tetrahydrofuran, and analyzed by adsorption HPLC (99:1, hexane:dioxane, 2 ml/min).

HPLC-purified retinol from a cauda epididymal tissue extract was evaporated, dissolved in 100 μl triethylamine, and reacted with 50 μl acetic anhydride (1 h, 50°C). Ethanol with 0.01% BHT (500 μl) and one volume of 4.25 M NaCl with 0.025 M NaOH were added. The retinyl acetate was extracted into petroleum ether (2 × 1 ml) and taken to dryness. The residues were dissolved in hexane and analyzed by adsorption HPLC (99:1 hexane:dioxane, 2.5 ml/min).

Retinyl ester peaks from a caput epididymal tissue extract were evaporated and then solubilized in 1% taurocholate in PBS (500 μl). Cholesterol ester hydrolase was added to 0.2 mg/ml and reacted 45 min at 37°C. After addition of one volume of ethanol with 0.01% BHT and NaCl (0.12 g), retinoids were extracted into petroleum ether (2 × 1 ml). After evaporation, residues were dissolved in 89:11 hexane:dioxane, and analyzed by adsorption HPLC (89:11 hexane:dioxane, 2 ml/min).

Assay of Epididymal Sperm for Retinol Esterification Activity

Caput epididymal sperm from five rats were purified by Percoll density centrifugation [14, 26]. The sperm suspension was diluted 1:10 with cold PBS and centrifuged (800 × *g*, 10 min). The pellet was homogenized in 4 vol ice-cold 0.10 M sucrose, 0.05 M KCl, 0.03 M EDTA, and 0.04 M potassium phosphate buffer, pH 7.2, at 12 000 rpm with a Polytron 3000. Protein was assayed using a Bio-Rad (Richmond, CA) protein assay according to instructions with BSA standards. To one of two aliquots (640-μg protein), iodoacetamide was added to a concentration of 1 mM and allowed to react with the protein (21°C, 10 min). Dithiothreitol was added to each sample to a concentration of 3 mM, BSA was added to a concentration of 40 μM, and CRBP purified from rat liver [27] complexed with 15-^{3}H-retinol [28] (15.3 Ci/mmol) was added to a concentration of 6 μM (500 μl final reaction volume). Reactions were allowed to proceed for 30 min at 37°C. Ethanol with 0.01% BHT (500 μl) was added. Retinyl esters were extracted with petroleum ether (2 × 1 ml), and fractionated by alumina chromatography [21, 22]. A control extraction of CRBP complexed with ^{3}H-labeled retinol was also performed. The ester fraction was analyzed by HPLC as described earlier. Fractions (0.25 ml) were collected, scintillation fluid was added, and tritium was counted as described earlier. The radioactivity present in HPLC fractions from a control reaction with heat-inactivated protein were subtracted.

RESULTS

Extraction Controls

Under the method employed here, no detectable retinoic acid was produced from control extractions of amounts of retinol that were in excess of endogenous retinol levels. Thus subsequent analyses of retinoic acid extracted from samples in which lesser amounts of retinol were present may be regarded with confidence.

Further, no detectable 9-*cis*-retinoic acid was produced from the control extraction of an amount of all-*trans*-reti-

TABLE 1. Rat epididymal tissue retinoid levels.

Retinoid	Caput	Corpus (pmol/g tissue)[a]	Cauda
Retinol[b]	2.7 ± 1.7 × 10^3	1.8 ± 0.8 × 10^3	1.7 ± 0.8 × 10^3
Retinyl ester[b]	6.2 ± 2.5 × 10^3	1.5 ± 0.7 × 10^3	1.4 ± 0.7 × 10^3
Retinoic acid[c]	13 ± 4	25 ± 5	35 ± 6
9-*cis*-Retinoic acid[d]	2.9 ± 1.0	3.8 ± 0.7	6.8 ± 0.9

[a]Averages of determinations ± SD).
[b]Six determinations from a total of 16 (caput) and 20 (corpus) rats and seven determinations from a total of 20 (cauda) rats (2–4 rats per determination).
[c]Three (corpus) or four (caput and cauda) determinations from a total of 16 (caput), 18 (corpus), or 18 (cauda) rats (2–6 rats per determination).
[d]Four (caput) or three (cauda) determinations from a total of 16 (caput), 18 (corpus), or 13 (cauda) rats (2–5 rats per determination).

noic acid that was in excess of endogenous levels. Thus the presence of 9-*cis*-retinoic acid in extracts from epididymal sources may be regarded with confidence as nonartifactual. Under the same conditions in control extractions, however, 10% of the all-*trans*-retinoic acid was isomerized to 13-*cis*-retinoic acid. Thus this isomer was presumed to be an artifact of the procedure and was quantified together with all-*trans*-retinoic acid.

Retinoid levels were corrected for recovery on the basis of extraction efficiency determinations.

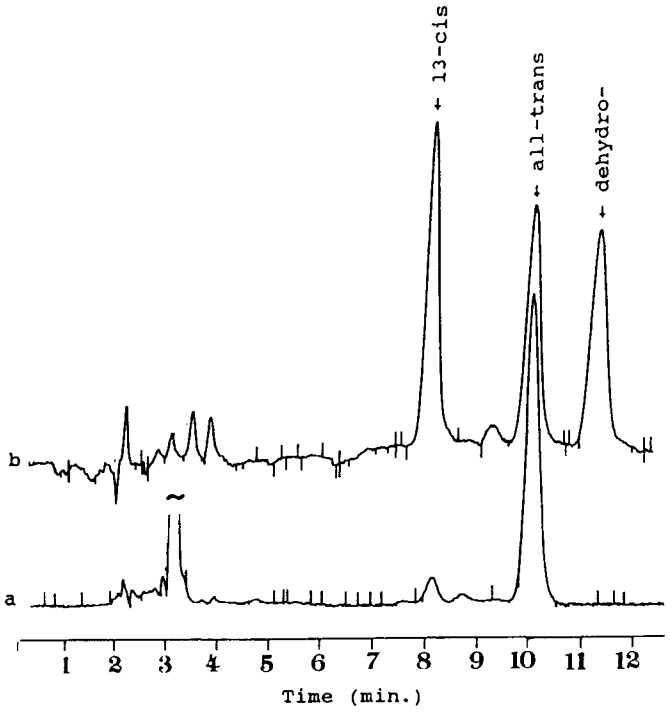


FIG. 1. Adsorption HPLC chromatogram showing presence of retinol in a rat cauda epididymal tissue extract (a). Standards of 13-*cis*-retinol, all-*trans*-retinol, and dehydroretinol are shown in the top tracing (b). Conditions were as described in *Materials and Methods*.

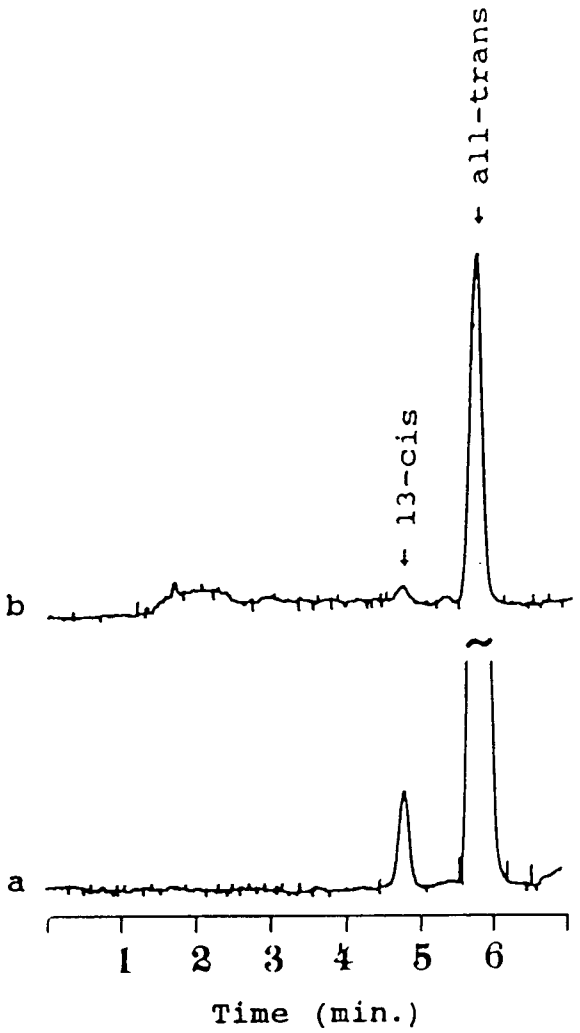


FIG. 2. Adsorption HPLC chromatogram of standards of 13-*cis*-retinyl acetate and all-*trans*-retinyl acetate (a) and of HPLC-purified all-*trans*-retinol from cauda epididymal tissue after acetylation (b). Conditions were as described in *Materials and Methods*.

Rat Epididymal Tissue Retinoid Analyses

Retinol (levels shown in Table 1) was present in rat epididymal tissue, as shown in Figure 1. The identities of the peaks assigned as retinol by HPLC retention times were further substantiated by derivatization as the acetate ester. Chromatography of the acetate ester derivative of an all-*trans*-retinol peak revealed only all-*trans*-retinyl acetate and a small amount of 13-*cis*-retinyl acetate (Fig. 2). Production of a small amount of the 13-*cis*-derivative from the all-*trans*-retinol peak suggested that other 13-*cis*-isomers observed in analyses were artifacts of extraction. No evidence was found for the presence of didehydroretinol in epididymis.

No significant difference between corpus and cauda tissue retinol levels was observed. Although the standard deviation for caput epididymal tissue retinol concentrations appeared to suggest no significant difference from corpus and cauda tissue retinol concentrations, a *t*-test suggested

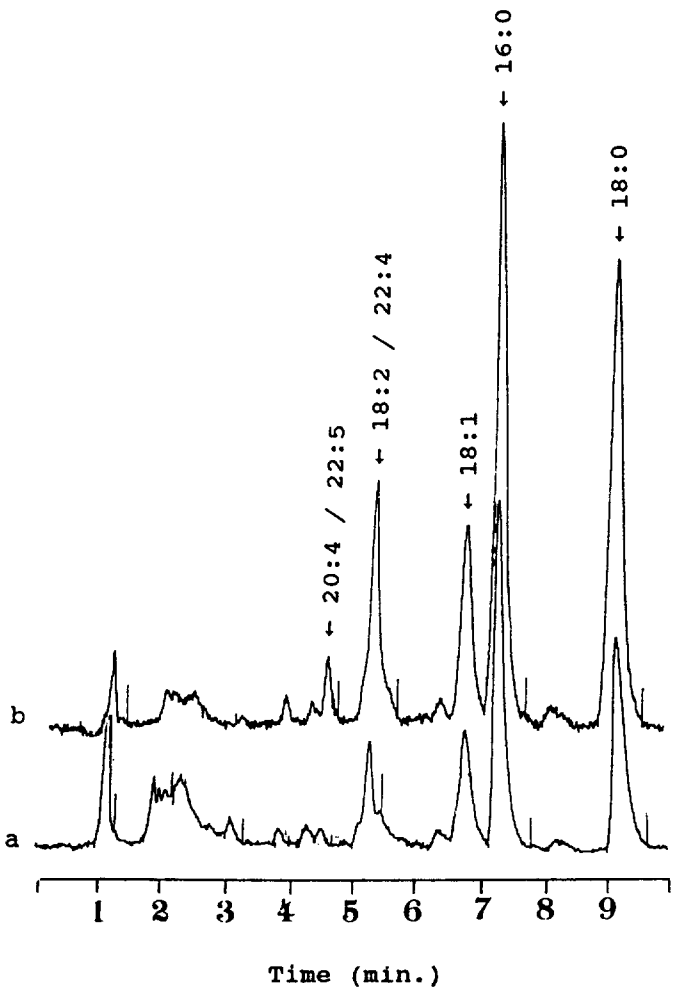


FIG. 3. Comparison of reverse-phase HPLC chromatograms showing presence of retinyl esters in rat vas deferens (a) and caput epididymal tissue (b). Numbers above the peaks designate the fatty acyl substituents. Conditions were as described in *Materials and Methods*.

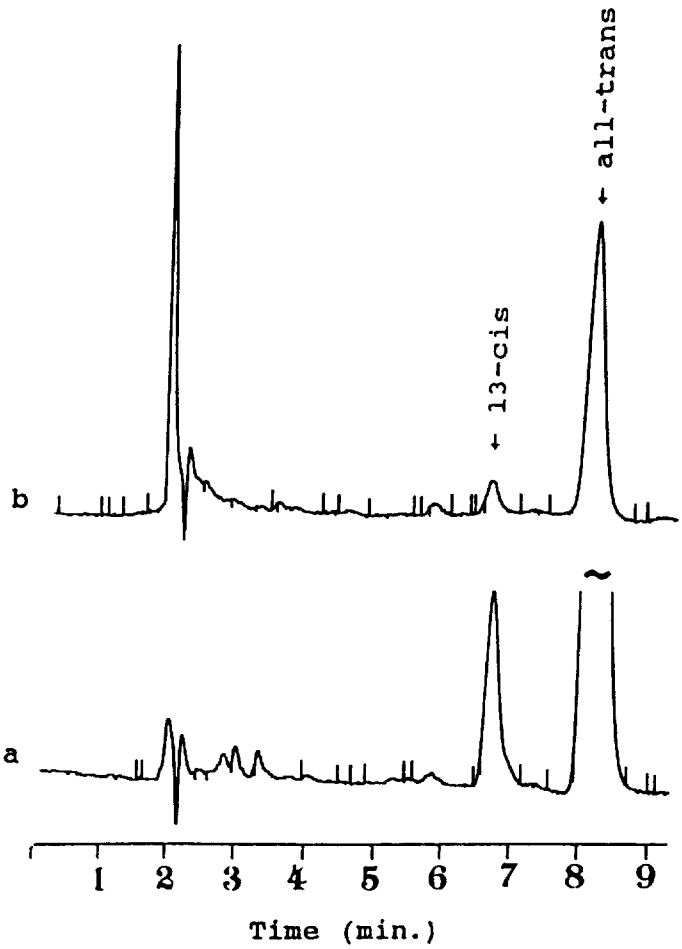


FIG. 4. Adsorption HPLC chromatograms of 13-*cis*- and all-*trans*-retinol standards (a) and of the enzymatic hydrolysate of HPLC-purified retinyl palmitate obtained from caput epididymal tissue (b).

that they were statistically different ($p < 0.05$) [29]. Thus, retinol levels appeared to be higher in caput than in corpus or cauda tissue.

Rat epididymal tissue also contained retinyl esters (Table 1, Fig. 3), which were at obviously higher levels in caput tissue than in the more distal epididymal segments. Retinyl ester levels did not appreciably differ between corpus and cauda tissues. The identities of the five major putative retinyl ester peaks observed in epididymal tissue were substantiated by enzymatic hydrolysis of the HPLC-purified retinyl ester peaks with porcine cholesterol ester hydrolase. Upon hydrolysis, retinol was recovered as the all-*trans*-isomer (91%-94%) and the 13-*cis*-isomer (6%-9%), as shown in Figure 4 for the palmitate derivative. Total retinol recovery was from 64% to 89% for the five ester peaks collected. Thus, the material may be regarded with confidence as representing retinyl esters.

The retinol fatty acylation patterns of the three major epididymal segments differed to some extent, though not markedly, as may be observed in Table 2. The major esters of retinol in all three segments were the palmitate (16:0) and stearate (18:0) esters. Retinyl oleate (18:1) and other esters were present in lesser amounts. Retinyl myristate (14:0) and palmitoleate (16:1), if present, were less than 1% of total esters. There was a slight trend toward higher proportions of retinyl oleate ester in cauda than in caput. Since retinyl linoleate (18:2) and retinyl docosatetraenoate (22:4) coeluted in the HPLC system described, ester material eluting with these standards was reported together. For the same reason, the arachidonate (20:4)/docosapentenoate (22:5) pair were reported together.

TABLE 2. Rat epididymal tissue retinyl acylation pattern.

Epididymal segment	Fatty acyl moiety[a]				
	20:4/22:5	18:2/22:4	18:1	16:0	18:0
Caput	2.2 ± 0.5	12 ± 1	12 ± 1	37 ± 1	37 ± 1
Corpus	1.0 ± 0.9	9.7 ± 1.5	16 ± 4	41 ± 4	32 ± 3
Cauda	1.4 ± 1.0	10 ± 3	17 ± 3	39 ± 4	32 ± 3

[a]Percent of total retinyl esters (±SD), from determinations as described in Table 1.

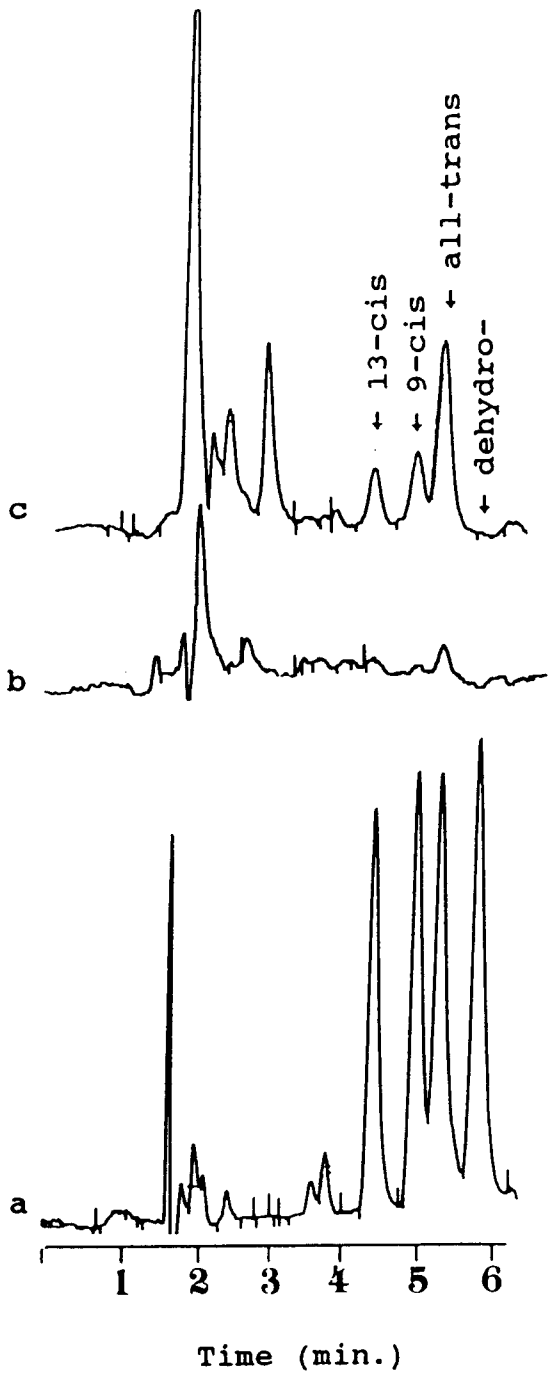


FIG. 5. Adsorption HPLC chromatogram of standards of dehydroretinoic acid and of three retinoic acid isomers (a), a chromatogram showing presence of retinoic acid in rat corpus and cauda epididymal spermatozoa (b), and a chromatogram showing the presence of retinoic acid isomers in corpus epididymal tissue (c). Conditions were as described in *Materials and Methods*.

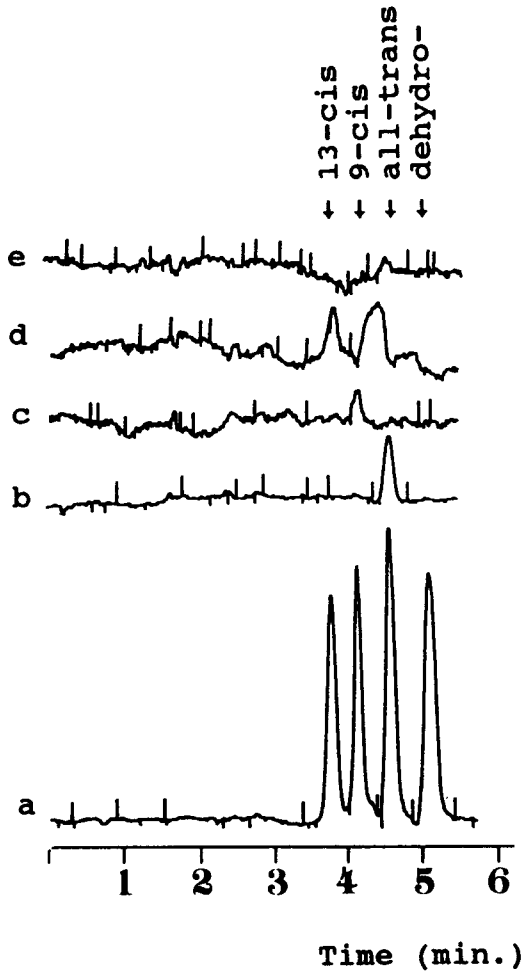
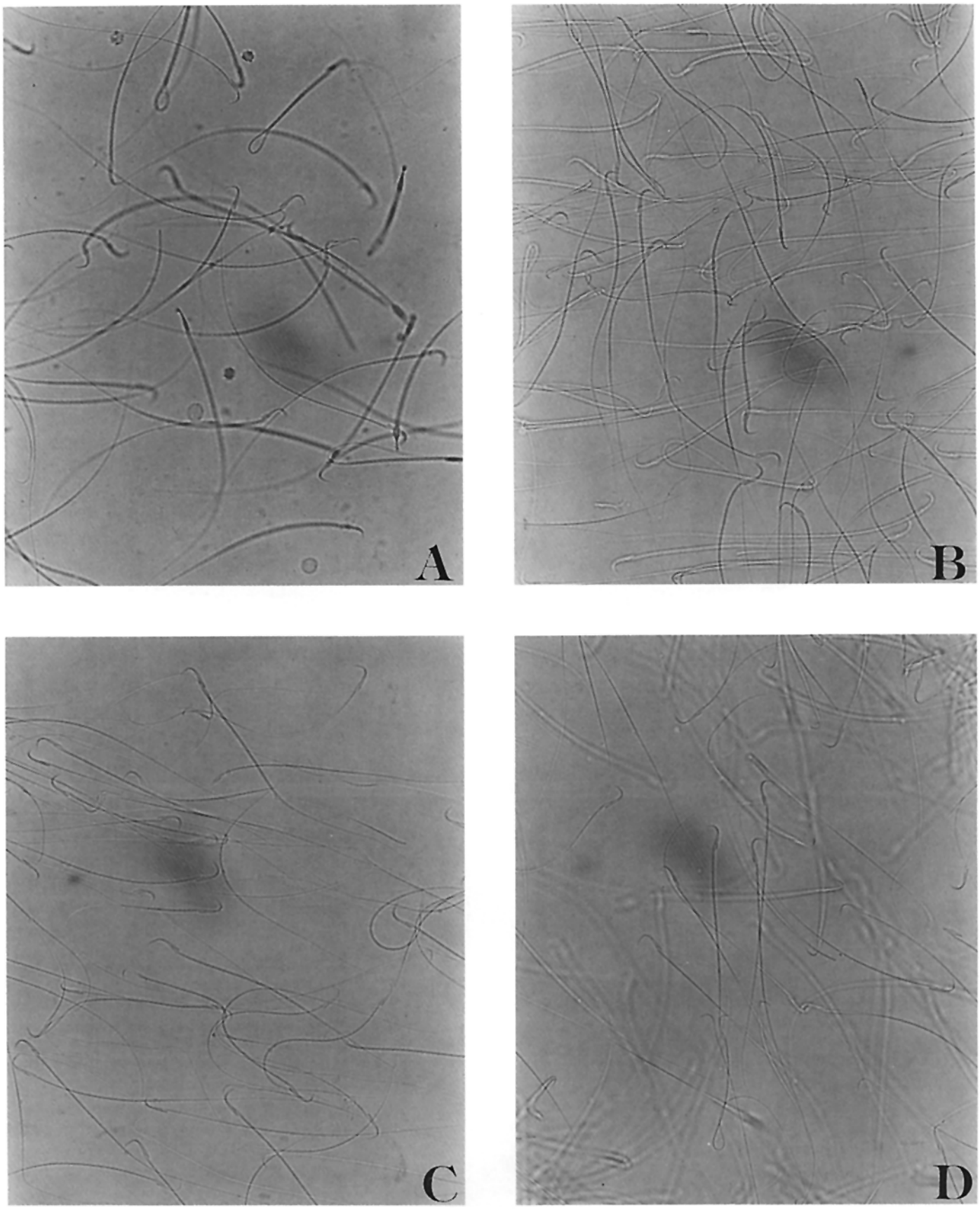


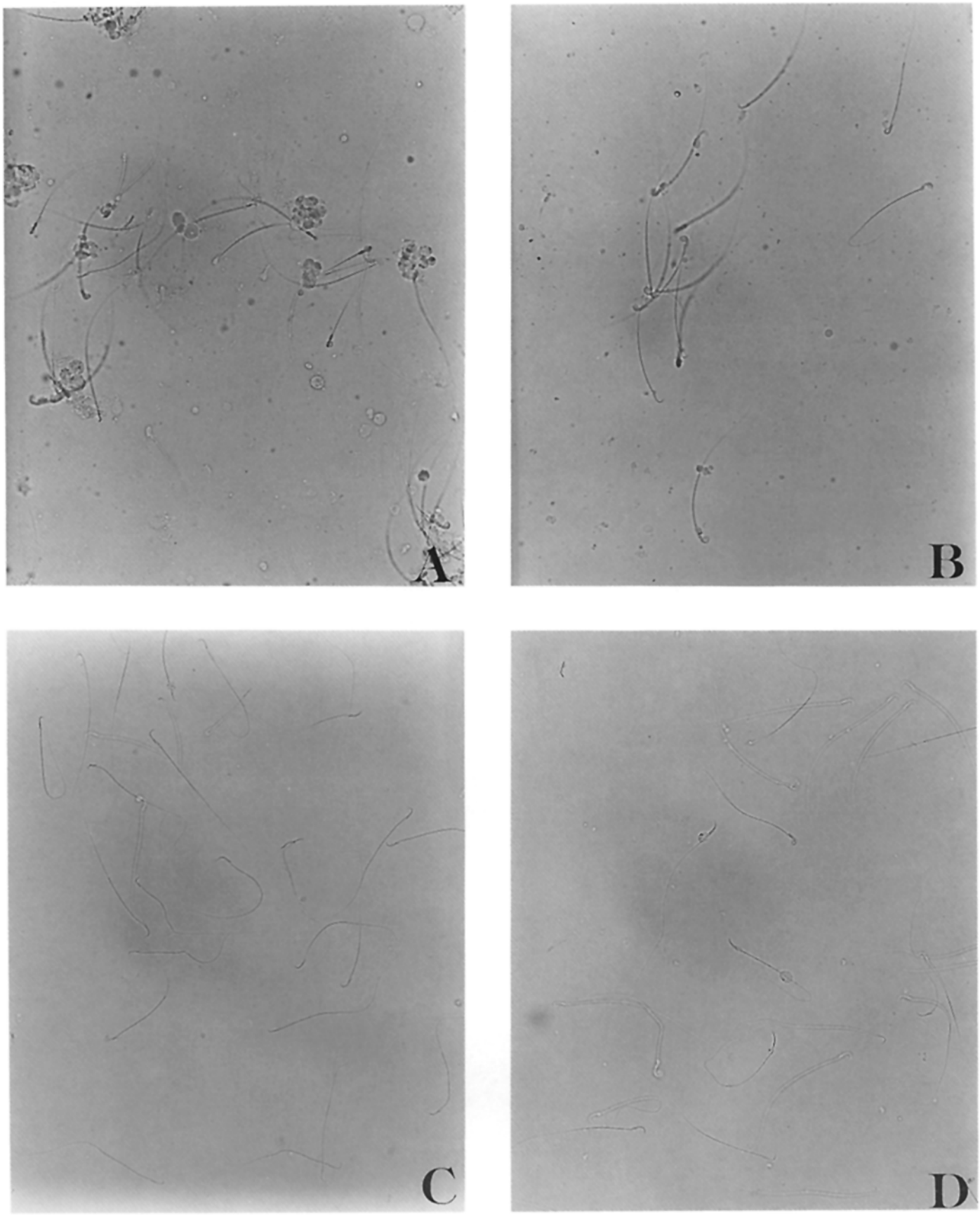
FIG. 6. Adsorption HPLC chromatograms of 13-*cis*-, 9-*cis*-, and all-*trans*-retinoic acid and dehydroretinoic acid methyl ester standards (a), and of the methyl ester derivatives of HPLC-purified all-*trans*-retinoic acid (b), 9-*cis*-retinoic acid (c), and 13-*cis*-retinoic acid (d) peaks obtained from a cauda epididymal tissue extract, and (e) a control extraction from a tube containing only diazomethane in tetrahydrofuran. The broad peak in (d) was presumed to be artifactual, as discussed in *Results*. Conditions were as described in *Materials and Methods*. Tracings were at 0.005 AUFS with attenuation in c, d, and e adjusted to half that in a and b.

The retinol fatty acylation pattern in epididymal tissue was very similar to that of vas deferens tissue (20:4/22:5 ester, 1.4%; 22:4/18:2 ester, 7.6%; 18:1 ester, 17%; 16:0 ester, 43%; 18:0 ester, 31%; one determination). Chromatograms of the retinyl ester fractions from a caput epididymal extract and the vas deferens extract are compared in Figure 3.

Rat epididymal tissue also contained retinoic acid (Table 1, Fig. 5). Retinoic acid isomer identity was substantiated by derivatization of the HPLC-purified isomers. Methyl ester derivatives of 13-*cis*-, 9-*cis*-, and all-*trans*-retinoic acid peaks eluted in a second adsorption HPLC system with retention

FIG. 7. Photomicrographs of filtered rat caput epididymal sperm obtained from a mince preparation before (A) and after (B) dextran wash; corpus and cauda sperm after dextran wash (C), and distal cauda sperm obtained by retrograde flush after dextran wash (D) showing presence of visible tissue contaminants before (A) and absence after (B-D) dextran wash. ×400.





times corresponding to the respective methyl ester standards (Fig. 6); thus assignment of the respective identities may be regarded with confidence. A second broad, irregularly shaped peak was noted in addition to the 13-*cis*-retinoic acid methyl ester derivative. The broad peak contained more integrated area than that of the original peak collected and thus was presumed to be artifactual. No didehydroretinoic acid was detected in epididymis.

Whole-tissue retinoic acid levels showed gradation of retinoic acid concentrations, with the lowest value observed in the caput, and the highest in the cauda (Table 1).

The putative ligand for the RXR receptors, 9-*cis*-retinoic acid [30, 31], was also identified in rat epididymal tissue (Figs. 5 and 6, Table 1) and showed an apparent gradation of concentrations from lowest levels in caput to highest in cauda.

Evaluation of Spermatozoal and Germ Cell Purity

Epididymal sperm preparations were carefully evaluated for purity to rule out the possibility of tissue contaminant contributions to spermatozoal retinoid analyses. Photomicrographs of rat epididymal sperm before and after dextran wash (Fig. 7) show elimination of visible cellular debris, and free cytoplasmic droplets, as observed by Tulsiani et al. [15], using the unmodified method. The use of a 15% dextran layer prevented formation of a packed pellet, thus enhancing spermatozoal preparation quality (greater than 90% heads and tails intact in all preparations).

Testis spermatozoa collected in the elutriator effluent suspension also contained free cytoplasmic droplets and possibly some small cytoplasts (Fig. 8B). The spermatozoa possibly included some late spermatids prematurely torn away from Sertoli cell attachment, which were indistinguishable from spermatozoa. Filtration through 20-μm Spectramesh filters allowed the free cytoplasmic droplets, cytoplasts, and debris to pass through the filter, while most spermatozoa were retained (Fig. 8, C and D), giving a clean preparation.

Rat Spermatozoal and Germ Cell Retinoid Analyses

Since the HPLC peaks of major interest in this study were confirmed as the assigned compounds from whole epididymal tissue extracts by derivatization, the respective spermatozoal compounds, identified by HPLC retention times, were presumed to be identical with those found in whole tissue.

Retinol levels in caput epididymal sperm were not determinable because of the neighboring elution of other material that strongly absorbed light at 325 nm, obscuring any potential retinol peaks. Retinol could be detected and quantified in corpus-plus-cauda sperm and in distal cauda sperm (Table 3). No difference in retinol level was noted between sperm from these segments.

TABLE 3. Rat spermatozoal retinoid levels.

Retinoid	Testis (enriched)	Caput	Corpus/cauda (pmol/10^9 sperm)	Distal cauda
Retinol	n.d.[a]	n.d.	30 ± 15[b]	24 ± 5[c]
Retinyl ester	140 ± 50[d]	110 ± 40[b]	26 ± 9[b]	3.3 ± 1.2[c]
Retinoic acid	n.d.	6.8 ± 1.9[e]	9.9 ± 4.5[b]	n.d.

[a]n.d. = Not determined.
[b]Average of three determinations (±SD) from a total of 30 rats (10 rats per determination).
[c]Average of four determinations (±SD) from a total of 65 rats (15–20 rats per determination).
[d]Average of determinations from three pairs of rats.
[e]Average of three determinations from a total of 40 rats (10–15 rats per determination).

Epididymal sperm contained considerable retinyl ester, more than of other retinoids until they reached the distal cauda. Retinyl ester content differed markedly among spermatozoal preparations from all segments examined, decreasing by orders of magnitude between proximal and distal epididymal segments. The decrease in retinyl esters was not due to loss of cytoplasmic droplets since there was little or no retention of cytoplasmic droplets attached to spermatozoa in preparations from caput epididymidis and less than 50% in corpus and cauda preparations. In particular, caput sperm contained essentially no cytoplasmic droplets, but had markedly higher retinyl ester levels than did corpus and cauda sperm. In no instance did we observe an effect of cytoplasmic droplet retention on retinyl ester levels.

Although acylation patterns of retinyl esters recovered from epididymal tissue segments showed little difference, the patterns of spermatozoal retinyl esters were position-dependent (Table 4). The proportion of the palmitate ester decreased, with a concurrent increase in the oleate ester for older sperm (Fig. 9).

TABLE 4. Characterization of spermatozoal retinyl acylation pattern.

Spermatozoal source	Fatty acyl moiety[a] 18:2/22:4	18:1	16:0	18:0
Rat testis (enriched)	—	—	100 ± 0[b]	—
Rat caput	7.5 ± 3.1	13 ± 3	43 ± 3	38 ± 3
Rat corpus/cauda	7.3 ± 2.7	21 ± 1	36 ± 3	36 ± 3
Human[c]	—	—	78 ± 4	22 ± 4

[a]Percent of total retinyl esters ± SD from determinations described in Table 3.
[b]No other esters detectable.
[c]In one of three determinations, 2% or less of the 14:0 and 18:1 esters was detected.

FIG. 8. Photomicrographs of filtered rat testis spermatozoa obtained from a mince preparation before (A) and after (B) centrifugal elutriation and after microfiltration with a 20-μm nylon mesh (C and D) showing progressive purification of testicular spermatozoa. Unattached droplets and small debris were essentially eliminated after microfiltration (C, D), but a few large cytoplasmic droplets remained attached to some spermatozoa (D). ×200.

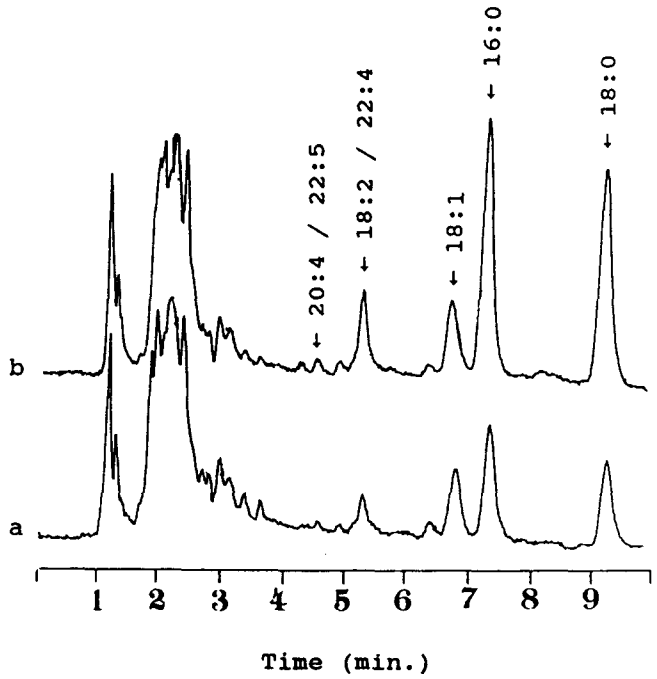


FIG. 9. Comparison of reverse-phase HPLC chromatograms showing retinyl ester patterns in rat corpus and cauda (a) and caput epididymal spermatozoal extracts (a). Numbers above the peaks designate the fatty acyl substituents. Conditions were as described in *Materials and Methods.*

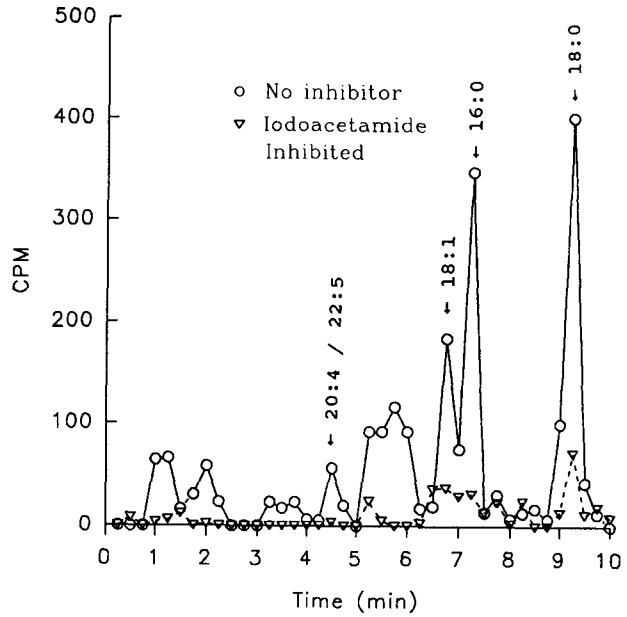


FIG. 10. Synthesis of ^{3}H-labeled retinyl esters by sperm homogenates. Reverse-phase HPLC chromatogram showing ^{3}H-labeled retinyl esters produced from retinol complexed with CRBP by caput epididymal sperm homogenates (solid line). The fatty acylation pattern was similar to that found endogenously in the sperm. The activity was inhibited by the sulfhydryl reagent iodoacetamide (broken line). Conditions were as described in *Materials and Methods.*

Testis spermatozoa also contained retinyl ester, consisting almost entirely of the palmitate ester. In addition, the ester levels were higher than levels observed in epididymal sperm (Table 4). Large cytoplasmic droplets remained attached to some testis spermatozoa (Fig. 8D), which could not be ruled out as contributors to the ester quantities detected. However, even if the bulk of the retinyl esters in testicular sperm were due to the few remaining large cytoplasmic droplets, it would not change the fact that the esters were of germ cell origin. If the esters detected were predominantly contained in the spermatozoa, then the higher levels reported for enriched testis sperm would suggest that rat spermatozoa possessed significant levels of retinyl esters before entering the epididymis, although acylated with a different acylation pattern than observed for the epididymal sperm.

An enriched spermatid preparation was found to contain retinyl esters in the form of 92% palmitate, 4% oleate, 3% myristate, and 1% stearate esters with 1.3×10^{4} total esters per 10^{9} round cells (one determination). The predominance of the palmitate ester was comparable to the acylation pattern observed for testis sperm preparations.

Rat epididymal spermatozoa also contained retinoic acid. The levels did not appear to differ significantly between caput and corpus-plus-cauda preparations. Possible 9-*cis*-retinoic acid peaks were visible in some chromatograms (Fig. 5) but could not be accurately quantified for the quantities of rat epididymal spermatozoa used in this study.

Presence of Retinol Esterification Activity in Epididymal Sperm

Homogenates of Percoll gradient purified caput epididymal sperm homogenates, free of cytoplasmic droplets, esterified ^{3}H-labeled retinol bound to CRBP (Fig. 10). HPLC analysis of the extracts revealed radioactivity eluting in peaks corresponding to retinyl esters, with an acylation pattern resembling that found endogenously in caput epididymal spermatozoa (Fig. 10). The activity that produced the ^{3}H-labeled esters was found to be almost completely abolished by 10-min preincubation with the sulfhydryl-blocking reagent, iodoacetamide (Fig. 10), a known inhibitor of lecithin-retinol acyltransferase, the primary retinol esterifying activity in numerous tissues, including testis. Boiled homogenates revealed only background counts in the retinyl ester region of the chromatogram, with the exception of a small artifactual impurity eluting in the 5.75-min fraction. This peak was found to occur in control extracts of the CRBP-^{3}H-retinol complex, which had not been exposed to spermatozoal homogenates.

Human Spermatozoal Retinoid Analyses

Human sperm contained considerable amounts of retinyl esters (160 ± 70 pmol/10^{9} sperm), about 50-fold more than seen for rat distal cauda sperm (Table 3). In addition, the ester pattern consisted of only retinyl palmitate and ret-

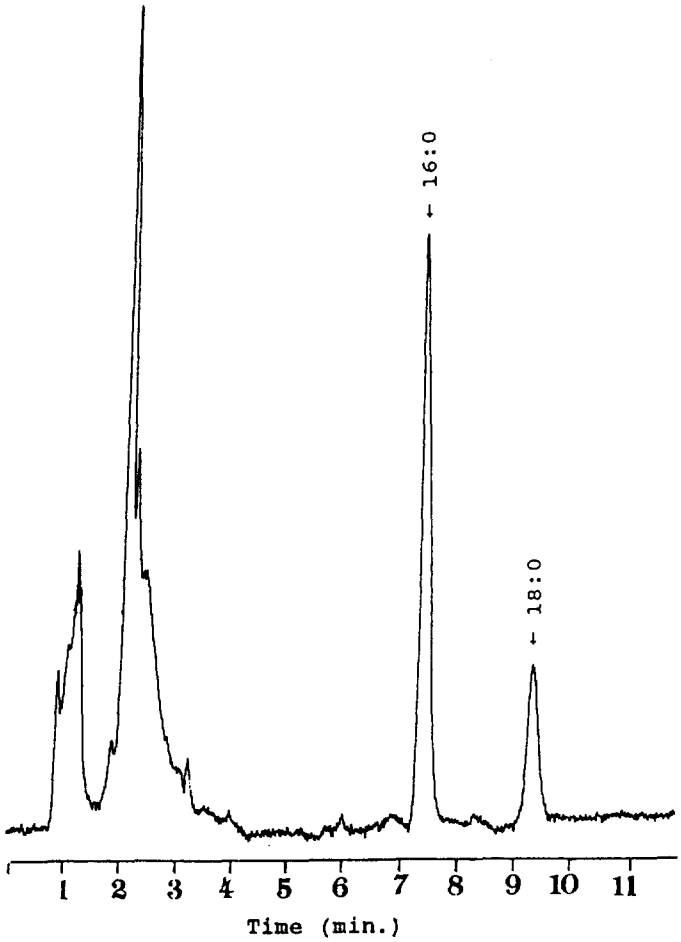


FIG. 11. Reverse-phase chromatogram of endogenous retinyl esters obtained from a human ejaculate spermatozoal extract showing presence of the palmitate (16:0) and stearate (18:0) esters. Conditions were as described in *Materials and Methods.*

inyl stearate (Fig. 11), in proportions different from those of rat spermatozoa (Table 4). Chromatograms from human sperm sample analyses had only suggestions of peaks representing either retinol or retinoic acid, which represented insufficient material for quantification.

DISCUSSION

The data reported here clearly demonstrate the presence of endogenous retinoids in several forms in rat epididymal tissue and in rat and human sperm. In all cases, identities were confirmed by derivatization of the HPLC-purified retinoids obtained from epididymal extracts. A previous study had indicated the presence of retinoids in spermatozoa [32], but the exact identities had not been determined. Of particular interest is the presence of graded concentrations of the two ligands for the RAR and RXR receptor families, all-*trans*-retinoic acid and 9-*cis*-retinoic acid, and the presence and subsequent loss of large quantities of retinyl esters by rat epididymal sperm that occurs during epididymal transit.

The increasing gradation of retinoic acid from caput to cauda in rat epididymis was as expected from RIA data for CRABP [4]. The determination here that two retinoic acid isomers were present in a graded fashion in epididymis also correlated with differences in expression of lumenal secretory proteins in the different segments of the epididymis [9]. This correlation may suggest that retinoic acid isomers, and possibly retinol, function in transcription enhancement of genes for proteins that are necessary for sperm maturation. Both all-*trans*-and 9-*cis*-retinoic acid have been shown to act via their nuclear receptors as transcriptional enhancement factors [33, 34].

Although retinol levels were found to be higher in caput than in distal epididymal segments, the distribution of CRBP [4, 5] and mRNA [35] (90% of both in the caput) suggested that a larger majority of retinol would be found in the caput than was observed.

Retinol levels in spermatozoa varied little between corpus-plus-cauda preparations and distal cauda preparations. However, these retinyl ester quantities were markedly lower than for caput spermatozoal preparations. In caput sperm preparations in which cytoplasmic droplets were not retained, retinyl esters were still present at the same levels as for preparations in which some cytoplasmic droplets were retained. Thus, loss of retinyl esters by sperm during epididymal transit cannot be explained by the normal loss of cytoplasmic droplets. Retinyl ester loss thus may be due to metabolism. The fact that the retinyl ester pattern differed between testis, caput, and corpus/cauda sperm might be explained by differential hydrolysis of the esters, as has been observed with brush border retinyl ester hydrolase. This enzyme hydrolyzes retinyl palmitate at faster rates than other retinyl esters [36]. Retinyl palmitate was the ester that had undergone greatest diminution from testis and caput to corpus and cauda spermatozoa. An alternate explanation is that the esters are continually hydrolyzed and re-esterified in a semi-equilibrium, with a net loss of retinyl esters as the sperm traverse the epididymis. This explanation is consistent with the fact that germ cells and testis sperm were shown to contain almost exclusively the palmitate ester of retinol, whereas caput epididymal sperm contained additional esters as described, which differed further in corpus/cauda preparations. Support for this possibility came from the finding of an epididymal spermatozoal retinol esterification activity, as reported here. Abolition of this esterification activity by the sulfhydryl reagent, iodoacetamide, was consistent with, though not proof of, the identity of the activity as a lecithin-retinol acyltransferase (LRAT), an activity that has been demonstrated to utilize a sulfhydryl group in the catalytic mechanism [37, 38]. Testicular germ cells are capable of esterifying retinol [39]. Thus, it may be that epididymal sperm have retained retinol esterification activity of germ cell origin. Since alteration of the sperm membrane phospholipid composition occurs during epididymal transit, and the sperm phospholipid acyl groups are con-

sistent with the acyl groups found esterified to retinol here [40, 41], the change in ester pattern might be explained by change in the LRAT phospholipid substrate fatty acid composition. Hydrolysis and re-esterification may be the more likely explanation of the changing retinyl ester acylation pattern, in the absence of evidence for transport of retinol from epididymal tissue to spermatozoa.

The presence of retinol and retinoic acid in spermatozoa obtained from distal epididymis in rats, and the finding of much higher levels of retinyl esters in human ejaculated spermatozoa certainly suggest a role for vitamin A in sperm function, given the fact that vitamin A must be provided to the developing spermatozoa by some active mechanism. Because spermatozoa are considered to be translationally inactive, a transcriptional enhancement function for retinol or its active metabolites in sperm is unlikely.

Several retinoic acid-responsive transcription factors derived from RAR and RXR genes have been found in *Xenopus laevis* eggs [42]. The RXR and RARα mRNA levels were found to increase dramatically in these cells upon fertilization and then to disappear abruptly between the blastula and gastrula stages. This implies roles for retinoids in gene expression immediately after fertilization. This phenomenon has not yet been investigated for rodents or other mammals. However, unless the sperm discards its vitamin A while moving through the female reproductive tract, it would be available to the rat and human ovum upon fertilization. Further work to elucidate the roles of vitamin A in reproductive physiology is necessary for more complete understanding of the findings reported here.

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